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An Investigation of Herbicide Interaction with the H^+-ATPase Activity of Plant Plasma Membranes

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Abstract: This study has investigated the activity of several herbicide classes at the plant plasma membrane. Two-phase partitioning was used to prepare highly purified plasma membrane vesicles from the monocotyledon weed black-grass (*Alopecurus myosuroides* Huds.) and the dicotyledon crop sugar beet (*Beta vulgaris* L. cv. Celt). The purity of the plasma membrane H^+-ATPase activity was characterised with respect to inhibitors, pH and substrate specificity. In both species, contamination of the plasma membrane by tonoplast fragments was largely eliminated and chlorophyll was absent. In addition, the plasma membrane H^+-ATPase from black-grass and sugar beet exhibited high vanadate sensitivity and a sharp pH profile around 6·5. Subsequently, H^+-ATPase activity was assayed in the presence (100 μM) and absence of four graminicide classes and auxin-type herbicides. Graminicides, including the aryloxyphenoxypropionate diclofop-methyl and the thiocarbamate triallate, inhibited H^+-ATPase activity by 50–80% in both species. However, other graminicides, including the cyclohexanediones and the chloroacetamide alachlor, had no affect. Similarly, auxin-type herbicides such as 2,4-D and MCPA did not inhibit H^+-ATPase activity. Results are discussed in relation to the proposed mode of action of these herbicides. © 1998 SCI

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Key words: black-grass (*Alopecurus myosuroides* Huds.); sugar beet (*Beta vulgaris* L.); plasma membrane; H^+-ATPase; graminicides

1 INTRODUCTION

Plantcell membranes form the outer boundary of the living protoplast and determine organelle compartmentation within the cell.[1] While the cell wall provides outer mechanical stability and protection, membranes regulate the internal chemical environment of the cytoplasm and organelles by acting as selective barriers to free solute movement. The plasma membrane forms the boundary between the highly organised and enzyme-rich cytosol and the cell wall and as such is important in the control of water, ion, nutrient and xenobiotic flow through the plant cell. These functions are under the control of membrane proteins and lipids which interact in a complex and, in most cases, still unknown fashion. However, the plasma membrane-bound ATPase (H^+-ATPase) has been comprehensively studied; it acts as a primary transporter by pumping protons out of the cell, creating pH and electrical potential differences across the membrane.[2] This enzyme, through its establishment of a transmembrane proton gradient, plays a key role in the regulation of multiple physiological functions, the transport of many solutes into and out of the cell, and auxin-induced growth.[3]

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Since many herbicides are lipophilic in nature and therefore preferentially partition into plant cell membranes, it is feasible that they may cause membrane disfunction or perturbation. Despite this proposition, few studies have directly investigated the effect of herbicides on plant cell membranes, although the topic has been reviewed.[4,5] Generally, membranes regulate herbicide uptake, can act as a possible site for primary mode of action, and are known to play an important role in the interaction between different herbicide classes.[6] In addition, a glutathione S-conjugate-dependent ATPase pump has been demonstrated on the tonoplast and is important in the transport of conjugated secondary products for storage in the vacuole.[7] Numerous studies have attempted to explain the mechanisms of herbicide uptake at the cellular level by using protoplasts or plasma membrane vesicles to allow for direct exposure of the membrane to an external solution.[8] A functional plasma membrane is important for herbicide uptake and subsequent accumulation at the target site to cause phytotoxicity.[9] Energy-dependent uptake is most likely due to the cell expending energy to maintain a pH gradient across the plasma membrane through the H^+-ATPase which utilises ATP as a primary substrate to pump H^+ out of the cell.[1] This has been shown in maize (*Zea mays* L.) where the uptake of imazethapyr was decreased by use of the plasma membrane H^+-ATPase inhibitors, vanadate and diethylstilbestrol, and the metabolic inhibitors, azide and N_2.[10] Similarly, sethoxydim uptake in wheat (*Triticum aestivum* L.) was reduced by both vanadate and diethylstilbestrol and increased by the fungal phytotoxin fusicoccin, a known plasma membrane H^+-ATPase activator, suggesting that uptake was dependent on ATP production and an operative H^+-ATPase.[11] No herbicide is known to act primarily as a direct inhibitor of plant cell membrane function. Graminicides, such as the aryloxyphenoxypropionates, are widely accepted as potent inhibitors of acetyl-CoA carboxylase, the first step in lipid biosynthesis. This is thought to be the primary mode of action, causing death by inhibiting the biosynthesis of new cell membranes in meristematic tissues.[12] However, there is no direct evidence to support this hypothesis and recently aryloxyphenoxypropionate-induced senescence initiated by an effect at or near the H^+-ATPase on the plasma membrane, resulting in the collapse of the transmembrane proton gradient, has been suggested to be of equal, if not more, importance to inhibition of acetyl-CoA carboxylase.[13]

Aryloxyphenoxypropionates, including diclofop-methyl, are incompatible with auxin-type herbicides and so cannot be tank-mixed for broad-spectrum use in the field.[14] The efficacy of diclofop-methyl is reduced on mixing with a broad-leaf herbicide such as the phenoxyalkanoic acid, 2,4-D.[15] Several theories regarding the mechanism of this antagonism have been proposed and some evidence points to an interaction at the cell membrane.[14] It has been suggested that aryloxyphenoxypropionates act as anti-auxins such that auxin-induced growth is antagonised and active membrane transport mediated through H^+-ATPase at the plasma membrane ceases. Similarly, bentazone and sethoxydim are two herbicides that complement each other in the control of a wide range of broad-leaved and graminaceous weeds in dicotyledonous crops. However, trials with this herbicide combination indicated a reduction in the efficacy of sethoxydim caused by a reduced uptake of the herbicide. Bentazone-inhibited uptake of sethoxydim was probably due to bentazone inhibition of plasma membrane activity from wheat leaves.[11]

Interestingly, it has recently been proposed that ATP-binding cassette (ABC) transporters, members of a protein superfamily located at the plasma membrane, are involved in detoxification processes.[16] In mammals, multiple-drug resistance (MDR) has been observed in tumour cells, in which overproduction of the ABC transporters causes active drug secretion. Thus, there is a decrease in intracellular drug concentration, target sites are not saturated and the cells become resistant to the drugs. The individual proteins associated with MDR have now been reported in various mammalian and invertebrate species, and more recently in yeasts and filamentous fungi, as recently reviewed by de Waard.[17] Indeed this author has speculated that overproduction of ABC transporter proteins in fungi may play a significant role in fungicide sensitivity. However, their overproduction and possible role in sensitivity to herbicides awaits demonstration.

Clearly, plant cell membranes, especially the plasma membrane, are important cellular sites that are proposed to play a role in herbicide uptake, mode of action and antagonism between different herbicide classes. The establishment of a transmembrane proton gradient through the integral membrane-bound enzyme H^+-ATPase appears to be central to all three. The objective of this study was to screen the effect of several graminicide classes and auxin-type herbicides directly on plasma membrane H^+-ATPase activity. Two-phase partitioning was used to prepare highly purified plasma membrane vesicles from leaf material of the monocotyledonous weed, black-grass (*Alopecurus myosuroides* Huds.) and the dicotyledonous crop, sugar beet (*Beta vulgaris* L. cv. Celt). The purity of the H^+-ATPase extracted was tested with respect to inhibitors, pH and substrate specificity to ensure that herbicide inhibition was due to an effect on the plasma membrane H^+-ATPase and not other membrane-bound H^+-ATPases.

2 MATERIALS AND METHODS

2.1 Plant material

Sugar beet, cv. Celt (British Sugar PLC, Norfolk, UK) was sown (3 seeds per pot) in John Arthur Bowers

potting compost in 100-mm diameter pots. Black-grass (Herbiseed Ltd, Berkshire, UK) was sown in trays containing the same medium. Plants were raised in glasshouse conditions (20–40°C day, 9–15°C night) at 60–70% RH. A photosynthetic flux density of 300–400 μmol m^{-2} s^{-1} was provided by natural daylight supplemented with high pressure sodium lamps and a 16-h photoperiod maintained throughout the study. The first true pair of sugar beet leaves and black-grass plants (one- to-two-leaf stage, lamina and sheath) were harvested approximately 15 days after sowing, frozen in liquid nitrogen and stored at −70°C until required.

2.2 Microsomal membrane preparation

All steps were carried out on ice. Frozen leaves were crushed to a fine powder, homogenised gently with scissors and then more vigorously with a pestle and mortar in sorbitol (230 mM), 2-[4-(2-hydroxyethyl)-piperazin-l-yl]ethanesulfonic acid (HEPES)-1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP) (50 mM, pH 8·0), potassium chloride (10 mM), ethylenedioxy bis(ethylenenitrilo)tetra(acetic acid) (EGTA; 3 mM) and dithiothreitol (DTT; 3 mM) added immediately prior to extraction. A ratio of 1 g tissue to 10 cm^3 homogenisation medium was used. The homogenate was filtered through four layers of muslin and centrifuged at 1000*g* for 5 min and then 10 000*g* for 10 min (MSE 21 centrifuge, Beckman Instruments). The resulting supernatant was carefully transferred to Oakridge-type centrifuge tubes and centrifuged at 50 000*g* for 45 min (L8-70 Ultracentrifuge, Beckman Instruments). The resulting microsomal membrane pellets were carefully resuspended in phase resuspension medium [sorbitol (350 mM) potassium phosphate (pH 7·8; 5 mM and DTT (2 mM)].

2.3 Two-phase partitioning

Aqueous two-phase partitioning was carried out as published.[18] Briefly, a microsomal membrane fraction prepared from 25 g of tissue, suspended in 0·4 ml of phase resuspension medium was added to form an 8 g phase system containing dextran T500 (62 g kg^{-1}), polyethylene glycol (MW 3350; 62 g kg^{-1}), sorbitol (350 mM), potassium chloride (5 mM), potassium phosphate buffer (pH 7·8; 5 mM) and DTT (1 mM). After mixing by inversion 40 times the phases were separated by centrifugation at 2500*g* for 5 min at 4°C. The procedure was performed for a total of three (sugar beet) or four partitions (black-grass) and the resulting upper and lower fractions were diluted approximately 10-fold in ATPase resuspension medium [sorbitol (350 mM), glycerol (100 g kg^{-1}), HEPES-BTP (pH 8·0; 2 mM) and DTT (2 mM)]. Following centrifugation at 100 000*g* for 45 min and suspension in ATPase resuspension medium, the membranes were frozen in liquid nitrogen and stored at −70°C until required.

2.4 Enzyme, protein and chlorophyll assays

H^+-ATPase activity was measured as the rate of liberation of orthophosphate (Pi) after incubation for 1 h at 37°C.[19] The standard reaction medium contained 2-hydroxy-1,1-bis(hydroxymethyl)ethylammonium (TRIS)-2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6·5, 40 mM), ammonium molybdate (0·1 mM), 'Triton' X-100 (0·05 ml $litre^{-1}$), magnesium sulfate (2 mM), potassium chloride (50 mM) and ATP-BTP (pH 6·5; 2 mM) in a reaction volume of 0·5 ml. H^+-ATPase activity was measured in the presence of magnesium and potassium having subtracted the activity occurring in the absence of these cations. Vanadate-sensitive H^+-ATPase activity attributable to the plasma membrane was measured in the presence of sodium vanadate (0·2 mM). H^+-ATPase activity attributable to the chloroplast (sodium azide sensitive, 1 mM) and tonoplast (potassium nitrate sensitive, 50 mM) was measured at pH 8·0. Approximately 5 μg membrane protein was included in each assay. The concentrations of Mg^{2+}, K^+ and 'Triton' X-100 used were optimised for H^+-ATPase activity in both species. Protein was determined using bovine serum albumin (BSA, Sigma Fraction V) as standard.[20] Chlorophyll was measured according to the formulae of Lichtenthaler and Wellburn.[21]

2.5 Herbicide

Herbicides were chosen to represent several graminicide classes, including aryloxyphenoxypropionates, cyclohexanediones, chloroacetamides and thiocarbamates. Auxin-type herbicides such as the phenoxyalkanoic acid 2,4-D were also studied. Other herbicides were examined as part of the overall screen. All herbicides (>95% purity; British Greyhound, Birkenhead, UK) were initially dissolved in acetone and then diluted with ATPase resuspension medium. The final concentration of acetone in the assay medium was 5 ml $litre^{-1}$ and did not affect H^+-ATPase activity (data not shown). Herbicides were added to the standard enzyme reaction medium for 30 min prior to starting the reaction with ATP-BTP (pH 6·5, 0·2 mM). Plasma membrane vesicles were therefore exposed to herbicides for a total of 90 min (including 1 h assay).

2.6 Statistical analysis

Data were analysed using Student's *t*-test to test the null hypothesis of zero mean difference at the 95% confidence level. Dose response curves were computed using the mathematics function in Slidewrite (Advanced Graphics Software Inc., Carlsbad, USA).

3 RESULTS

3.1 Characterisation of H^+-ATPase activity in the upper and lower membrane phases

The specific activity of H^+-ATPase measured in the membrane phases varied from different batches of plants. Generally, H^+-ATPase activity in the upper phase ranged from 40 to 90 μmol mg^{-1} protein h^{-1} in black-grass and 20 to 40 μmol mg^{-1} protein h^{-1} in sugar beet, but was always much greater than that observed in the microsomal membrane fraction. The H^+-ATPase activity in the upper phase was very sensitive to inhibition by vanadate, accounting for almost 70% inhibition in both black-grass and sugar beet, compared to approximately 30% in the lower phase (Table 1). Azide had no effect on the H^+-ATPase activity in the upper phase in both species, whereas inhibition in the lower phase accounted for about 30% in black-grass and sugar beet. Nitrate gave a similar level of inhibition to both vanadate and azide in the lower phase (approx. 30% in both species), but, although the extent of inhibition by nitrate was reduced in the upper phase, it still accounted for 13% (black-grass) and 5% (sugar beet) inhibition of the H^+-ATPase activity (Table 1). In addition, no chlorophyll was detected in the upper phase of either species (data not shown). The H^+-ATPase activity in the upper phase of both black-grass and sugar beet showed a sharp pH profile with an optimum between pH 6 and 6·5 (Fig. 1). The upper phase of black-grass showed a high degree of substrate specificity for ATP at pH 6·5 than at pH 8·0, whereas in the lower phase, hydrolysis of other nucleoside triphosphates at both pH 6·5 and pH 8·0 was similar to that of ATP, particularly GTP and ITP (Table 2). Summarising these observations and comparing them to enzyme sensitivities widely reported in the literature, these data imply that the upper phase was enriched in plasma membrane vesicles and lacking in contamination from other membranes, especially of tonoplast, chloroplast and mitochondrial origin.

TABLE 1
Effect of Various Inhibitors of H^+-ATPase Activity on the Upper and Lower Phase of a Sugar Beet and Black-Grass Microsomal Membrane Fraction after Two-Phase Partitioning

	Inhibition of H^+-ATPase activity (%)	
Species and reagent	*Upper phase*	*Lower phase*
Sugar beet:		
Vanadate (0·2 mM)	67	29
Azide (1·0 mM)	0	31
Nitrate (50 mM)	5	26
Black-grass:		
Vanadate (0·2 mM)	68	27
Azide (1·0 mM)	0	30
Nitrate (50 mM)	13	34

Data were calculated from the means of three separate lower and upper phases from each species. Each enzyme from each phase was assayed three times.

TABLE 2
Substrate Specificity of H^+-ATPase Activity at pH 6·5 and pH 8·0 in the Upper and Lower Phases after Two-Phase Partitioning of a Black-Grass Microsomal Membrane Fraction

	H^+-ATPase activity (μmol mg^{-1} protein h^{-1})(±SEM)			
	pH 6.5		*pH 8.0*	
Substrate	*Upper*	*Lower*	*Upper*	*Lower*
ATP	41·65 (±2·70)	17·99 (±0·49)	10·95 (±0·23)	14·79 (±0·67)
GTP	4·08 (±0·34)	16·74 (±0·21)	4·46 (±0·18)	19·83 (±0·34)
ITP	2·20 (±0·22)	13·66 (±0·31)	3·46 (±0·27)	17·55 (±0·60)
ADP	1·68 (±0·43)	2·63 (±0·09)	0·00	1·04 (±0·16)
IDP	0·39 (±0·25)	1·18 (±0·06)	0·00	0·64 (±0·14)
AMP	0·00	0·00	0·00	0·00
PNP	0·00	0·00	0·00	0·26 (±0·15)
GP	0·00	0·00	0·00	0·00
PPi	9·77 (±0·57)	5·88 (±0·40)	14·14 (±0·41)	8·08 (±0·14)

Data were calculated from means (±SE) of three separate lower and upper phases. Each assay was conducted in triplicate. Substrates were added at 2·0 mM and included adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), inosine 5′-triphosphate (ITP), adenosine 5′-diphosphate (ADP), inosine 5′-diphosphate (IDP), adenosine 5′-monophosphate (AMP), bis(*p*-nitrophenyl)phosphate (PNP), *β*-glycerophosphate (GP) and pyrophosphate (PPi).

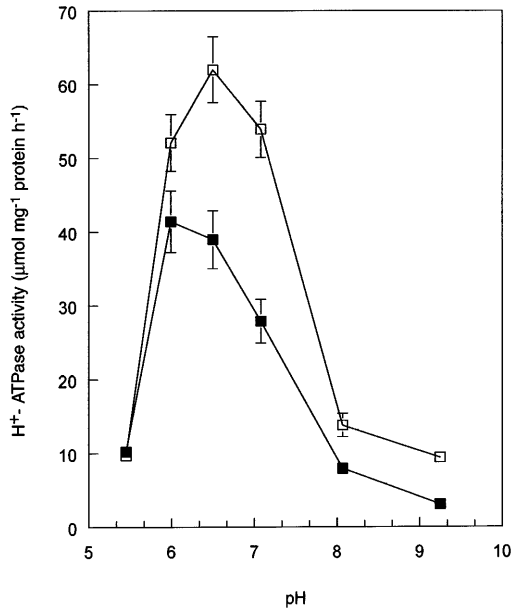

Fig. 1. pH dependency of H^+-ATPase activity in the upper phase of (■) sugar beet and (□) black-grass after two-phase partitioning. Each point represents the mean specific activity (µmol mg^{-1} protein h^{-1}) of three separate upper phases, each assayed in triplicate. Bar = SEM.

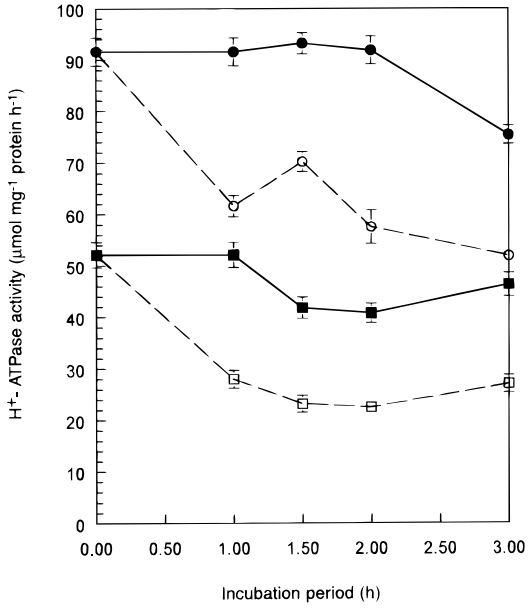

Fig. 3. The effect of incubating isolated plasma membrane vesicles with diclofop-methyl for various times prior to assay of H^+-ATPase activity. Closed symbols represent control, untreated activities (■, □ sugar beet; ●, ○ black-grass), whereas open symbols represent activities after treatment with 100 µM diclofop-methyl. Bar = SEM of three separate upper phases, each assayed in triplicate.

3.2 Herbicide screen

The specific activities of H^+-ATPase of the upper phase of black-grass and sugar beet in the presence and absence of a range of graminicides, auxin-type herbicides and representatives of other herbicide classes are shown in Table 3. At 100 µM, aryloxyphenoxypropionates such as diclofop-methyl and haloxyfop-methyl inhibited H^+-ATPase activity by approximately 50% and 40% in sugar beet and black-grass, respectively ($P < 0{\cdot}05$). Diclofop-acid was far less active than its methyl-ester. The thiocarbamates, diallate and triallate, inhibited H^+-ATPase activity to a greater extent than the aryloxyphenoxypropionates, 70% and 80% respectively in sugar beet ($P < 0{\cdot}001$). However, a third thiocarbamate, EPTC, had no effect in either species ($P > 0{\cdot}05$). Cyclohexanediones were inactive at 100 µM, except that cycloxydim inhibited H^+-ATPase activity by 30% in sugar beet ($P < 0{\cdot}001$). The auxin-type herbicide 2,4-D slightly stimulated H^+-ATPase activity in sugar beet ($P < 0{\cdot}05$), but generally this herbicide class had no effect on H^+-ATPase activity in either species ($P > 0{\cdot}05$). Trifluralin and phenmedipham inhibited H^+-ATPase activity in both species by 15–25% ($P < 0{\cdot}001$), whereas bentazone inhibited activity by 15% in sugar beet alone ($P < 0{\cdot}001$). Chlorotoluron slightly activated the H^+-ATPase activity in black-grass ($P < 0{\cdot}05$) but had no effect in sugar beet ($P > 0{\cdot}05$). The imidazolinone, imazethapyr, the sulfonylureas chlorsulfuron and metsulfuron-methyl and metamitron at 100 µM had no significant effect on H^+-ATPase activity *in vitro* in either black-grass or sugar beet ($P > 0.05$).

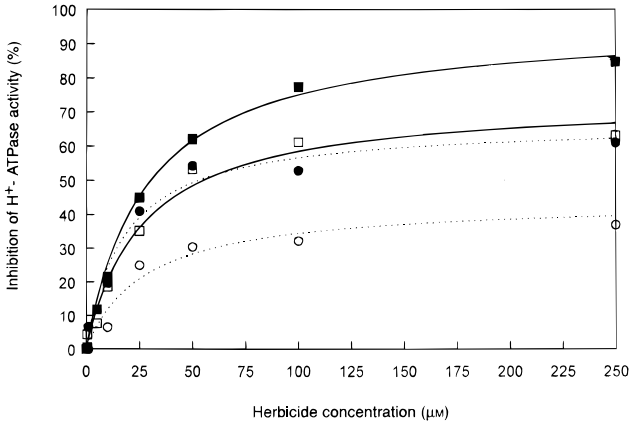

Fig. 2. The inhibition of plasma membrane H^+-ATPase activity extracted from sugar beet (closed symbols) and black-grass (open symbols) by (■, □) triallate and (●, ○) diclofop-methyl. Data were calculated from means of three enzyme assays each conducted on three separate plasma membrane fractions from both species.

3.3 Herbicide dose-response curves

In both species, herbicides inhibited H^+-ATPase activity in a dose-dependent manner over the range 0–250 µM, as illustrated for triallate and diclofop-methyl (Fig. 2). Generally, maximum inhibition was observed at 50–100 µM with no further inhibition at 250 µM. A proportion of the measured H^+-ATPase activity, for example 20% with triallate in sugar beet, was not inhibited by herbicides. The concentration of triallate

TABLE 3
Effect of Herbicides from Representative Classes on H^+-ATPase Activity on the Upper Phase of Sugar Beet and Black-Grass Microsomal Membrane Fractions after Two-Phase Partitioning

Herbicide	*Class*	*Change in H^+-ATPase activity after 30 min incubation with 100 μM herbicide (%)*[a]	
		Sugar beet	*Black-grass*
Triallate	Thiocarbamate	−80 (0·001)	−63 (0·001)
Diallate	Thiocarbamate	−70 (0·001)	−49 (0·001)
EPTC	Thiocarbamate	−3 (NS)	−3 (NS)
Diclofop-methyl	Aryloxyphenoxypropionate	−54 (0·001)	−37 (0·001)
Diclofop-acid	Aryloxyphenoxypropionate	−19 (0·01)	−8 (NS)
Haloxyfop-methyl	Aryloxyphenoxypropionate	−46 (0·001)	−35 (0·001)
Fenoxaprop-ethyl	Aryloxyphenoxypropionate	−22 (0·001)	−12 (0·001)
Quizalofop-ethyl	Aryloxyphenoxypropionate	−21 (0·001)	−25 (0·001)
Cycloxydim	Cyclohexanedione	−29 (0·001)	−4 (NS)
Sethoxydim	Cyclohexanedione	−1 (NS)	−7 (NS)
Alachlor	Chloroacetamide	−9 (NS)	−5 (NS)
2,4-D	Auxin-type	+12 (0·05)	+5 (NS)
Mecoprop	Auxin-type	−7 (0·05)	−2 (NS)
Dichlorprop	Auxin-type	−1 (NS)	0 (NS)
Clopyralid	Auxin-type	−10 (NS)	+3 (NS)
Fluroxypyr	Auxin-type	−6 (NS)	+1 (NS)
MCPA	Auxin-type	+3 (NS)	−1 (NS)
Trifluralin	2,6-dinitroaniline	−15 (0·001)	−14 (0·001)
Phenmedipham	Phenyl-carbamate	−24 (0·001)	−10 (0·01)
Bentazone	Benzothiadiazole	−15 (0·001)	+2 (NS)
Chlorotoluron	Urea	−5 (NS)	+5 (0·05)
Metamitron	1,2,4-triazinone	+1 (NS)	+2 (NS)
Chlorsulfuron	Sulfonylurea	−1 (NS)	−5 (NS)
Metsulfuron-methyl	Sulfonylurea	+3 (NS)	−5 (NS)
Imazethapyr	Imidazolinone	−1 (NS)	−1 (NS)
Control activity (μmol mg^{-1} protein h^{-1}) (±SE)		39·05 (±1·15)	40·55 (±2·01)

[a] Figures in parentheses represent level of statistical significance. NS = not significant.
Data were calculated from means of three enzyme assays each conducted on three separate upper phases from sugar beet and black-grass.

TABLE 4
Herbicide Concentrations Required to Inhibit H^+-ATPase Activity by 50%

Herbicide	*Concentration required to inhibit H^+-ATPase activity of 50% (μM) (±SEM)*	
	Sugar beet	*Black-grass*
Diclofop-methyl	17·0 (±2·00)	30·0 (±3·00)
Haloxyfop-methyl	11·8 (±3·00)	24·7 (±2·60)
Diclofop-acid	124·7 (±1·24)	ND[a]
Triallate	14·6 (±2·98)	16·6 (±2·17)
Diallate	48·0 (±2·93)	70·6 (±4·30)

[a] ND = not determined.
Data were calculated as means (±SE) from dose response curves similar to those shown in Fig. 2

required to reduce maximum H^+-ATPase inhibition by 50% was 15 μM in sugar beet, similar to that calculated for black-grass, 17 μM (Table 4; $P > 0{\cdot}05$). However, the concentrations of diclop-methyl and haloxyfop-methyl needed to reduce maximum H^+-ATPase activity inhibition by 50% were lower in sugar beet than in black-grass ($P < 0{\cdot}05$). Similarly in sugar beet, H^+-ATPase activity was more sensitive to the methyl ester of diclofop than the acid form ($P < 0{\cdot}05$).

3.4 Time course of herbicide inhibition

In both species, maximum inhibition of H^+-ATPase activity was observed after 60 min incubation with diclofop-methyl (Fig. 3). No further inhibition or recovery of activity was observed up to 180 min incubation in either black-grass or sugar beet.

4 DISCUSSION

The initial objective of this study was to use two-phase partitioning to extract plasma membrane vesicles of sufficient purity from green leaves of a monocotyledonous weed (black-grass) and a dicotyledonous crop (sugar beet) to permit the determination of the effect of different graminicide classes and auxin-type herbicides on H^+-ATPase activity *in vitro*. The methods used largely followed those previously published for sugar beet, except that a second slow centrifugation step was introduced in the extraction procedure.[22] This reduced the amount of chlorophyll and therefore contamination by thylakoid membranes in the microsomal membrane fraction prior to two-phase partitioning. Reichers *et al.*[8] adapted the same technique to isolate plasma membrane from fat hen (*Chenopodium album* L.) and virtually the same method has been used in the present study to isolate enriched plasma membrane vesicles from black-grass, albeit using a greater number of partition steps of two-phase partitioning. This was necessary since a significant proportion of chlorophyll was present in the upper phase from black-grass after the third partition.

It was essential that the purity and quantity of the isolated plasma membrane was known before the effect of herbicides on the membrane-bound H^+-ATPase could be assessed. The concept of enzyme markers is well established and it is generally accepted that vanadate-, azide- and nitrate-sensitive H^+-ATPase activities are good markers for the plasma membrane, chloroplast and tonoplast, respectively.[23] Vanadate sensitivity accounted for 70% of the H^+-ATPase activity at pH 6·5 in both species (Table 1). This was comparable to an earlier study using wheat, in which vanadate sensitivity of the upper phase accounted for 72% of the H^+-ATPase activity measured.[11] In both black-grass and sugar beet, vanadate showed a stronger inhibition of the H^+-ATPase activity in the upper phase than in the lower phase. Two-phase partitioning largely eliminated contamination of the plasma membrane by the chloroplast because of a lack of measurable chlorophyll and also the known chloroplast H^+-ATPase inhibitor, azide, had no effect on the H^+-ATPase activity. In both species, there was slight inhibition by nitrate in the upper phase, suggesting some tonoplast contamination. However, azide and nitrate inhibition of H^+-ATPase activity were much greater in the lower phases of both black-grass and sugar beet, suggesting that the chloroplast and tonoplast membranes were predominant in the lower phase. The purity of the plasma membrane was further studied by measuring the pH optimum of the H^+-ATPase activity (Fig. 1). The H^+-ATPase activity in the upper phases of both species showed a sharp pH profile with an optimum occurring between pH 6 and 6·5. High vanadate sensitivity and a sharp pH optimum around 6·5 are general properties of the plasma membrane H^+-ATPase.[2] This is clearly demonstrated by the characteristics of the H^+-ATPase activity associated with the upper phases of both species. In addition, substrate specificity was also examined in the upper phase from black-grass (Table 2). There was a high degree of specificity for ATP at pH 6·5 but not at pH 8·0 in the upper phase from black-grass and notable pyrophosphatase activity was also observed. The tonoplast and chloroplast H^+-ATPases are generally accepted to have pH optima of 8·0 and from this evidence do not appear to be present in the upper phase in a large amount.[23] A measurable pyrophosphatase activity in the upper phase, indicative of contamination by tonoplast vesicles, has been observed before in plasma membrane fractions isolated from castor bean (*Ricinus communis* L.).[24] In the lower phase, there were similar specificities for ATP, GTP and ITP at both pH 6·5 and pH 8·0, suggesting a more mixed enzyme, and therefore membrane, population. Any effect of herbicides on H^+-ATPase in the upper phase of either black-grass or sugar beet will therefore be due to an effect on the plasma membrane H^+-ATPase. Subsequently, activity in the presence and absence of different herbicide classes was measured.

Herbicides could be ranked according to their ability to inhibit H^+-ATPase activity of isolated plasma membrane vesicles in both sugar beet and black-grass: thiocarbamate > aryloxyphenoxypropionate > cyclohexanedione > chloroacetamide > auxin-type (Table 3). The enzyme from the dicotyledon sugar beet was generally more sensitive to herbicide inhibition than that from the monocotyledon black-grass, although the significance of this observation is unclear. Trifluralin, phenmedipham and bentazone also inhibited H^+-ATPase activity *in vitro*, but imazethapyr, chlorsulfuron, metsulfuron-methyl and metamitron were inactive under the conditions described. Inhibition by trifluralin and phenmedipham was expected, since

both function as uncouplers.[6] Similarly, an effect of bentazone at the plasma membrane has been predicted.[25]

The two thiocarbamates, triallate and diallate, inhibited H^+-ATPase activity up to 80% of untreated control in sugar beet at 100 μM, whilst a third thiocarbamate, EPTC, had no effect in either species. The concentrations of triallate required to inhibit H^+-ATPase activity by 50% were 15 and 17 μM in sugar beet and black-grass, respectively, suggesting that activity was highly sensitive to inhibition by triallate (Table 4). Even at 5 μM triallate, a 12% inhibition of H^+-ATPase activity was observed in sugar beet (Fig. 2). Maximum inhibition (60–80%) of H^+-ATPase activity was recorded at 100 μM, with no further inhibition at higher concentrations. Consequently, there appeared to be a proportion of H^+-ATPase activity that could not be inhibited by herbicide treatment. However, since H^+-ATPase activity attributable to the plasma membrane was defined as that exhibiting sensitivity to vanadate, which in this study accounted for 70% of the H^+-ATPase activity in the upper phase from both species, it is proposed that inhibition of plasma membrane H^+-ATPase activity by triallate and diallate was virtually complete at 100 μM.

The primary site of action of the thiocarbamates is not known. They appear to inhibit the biosynthesis of very long chain fatty acids as a direct inhibition of acetyl-CoA elongases in epidermal cells, and so alter the quality and quantity of leaf-surface lipids, leaving the plants vulnerable to environmental stress.[12] An inhibition of protein synthesis has also been observed.[12,26–28] More recently, thiocarbamates have been shown to inhibit gibberellic acid biosynthesis in cell-free enzyme preparations from sorghum (*Sorghum bicolor* L.).[29,30] Evidence that the inhibition of surface lipid biosynthesis is the primary mode of action is largely indirect, and it is unlikely that impaired synthesis alone would be phytotoxic.[31] An effect at the plasma membrane, particularly a rapid inhibition of H^+-ATPase activity, may make a major contribution to the herbicidal activity of these molecules. We speculate that if plasma membrane H^+-ATPase activity is inhibited, then the export of epicuticular wax intermediates from epidermal cells to the cuticle may be impaired.

It is unclear why EPTC did not inhibit H^+-ATPase activity, but there is some evidence to suggest that the sulfoxide metabolite of EPTC may be more herbicidally active than the parent molecule.[32] EPTC may inhibit the synthesis of acetyl Co-A and perhaps EPTC sulfoxide is required to inhibit H^+-ATPase activity whilst EPTC alone is inactive.[33] In addition, diallate is reported to be more effective at inhibiting gibberellic acid biosynthesis than EPTC.[30] The addition of a chlorine atom (two in diallate, none in EPTC) is thought to increase thiocarbamate activity greatly and could explain the greater inhibition of H^+-ATPase activity both by diallate and by triallate, which has three chlorine atoms per molecule. This may also explain why, in both species, the plasma membrane was more sensitive to triallate than to diallate. The chloroacetamide, alachlor, has many similarities to the thiocarbamates, including type of application, efficacy and selectivity, although its precise mode of action is also uncertain.[34] It has been demonstrated that micromolar concentrations of acetochlor may also inhibit fatty acid biosynthesis.[35] In the present study however, alachlor at 100 μM did not inhibit H^+-ATPase activity in either sugar beet or black-grass, suggesting that chloroacetamides are inactive at the plasma membrane.

Aryloxyphenoxypropionates also inhibited plasma membrane H^+-ATPase activity in both species, with the methyl esters more effective than the ethyl esters or the free acid. Inhibition was similar to the thiocarbamates in that a proportion of H^+-ATPase activity could not be inhibited by the aryloxyphenoxypropionates. However, since only 70% of the measured H^+-ATPase activity was vanadate-sensitive, it may be concluded that a large proportion of H^+-ATPase activity attributable to the plasma membrane was inhibited by diclofop-methyl at 100 μM. The mechanism of action of aryloxyphenoxypropionates has been a topic of controversy owing to the absence of definitive evidence implicating a specific mechanism. With few exceptions, the *in-vitro* inhibition of acetyl-CoA carboxylase correlates with whole-plant responses to aryloxyphenoxypropionates.[36] However, it is not certain whether a limitation of the supply of fatty acids alone in intact plants is the cause of whole-plant responses and the rapid lethality observed in susceptible species treated with diclofop-methyl and other analogues. Most responses appear to be accountable by a mechanism involving diclofop-methyl-induced senescence that may be initiated by an effect on the plasma membrane.[13] Inhibition of the integral membrane protein H^+-ATPase would result in the collapse of the transmembrane proton gradient.[13] This inhibition appears to be irreversible, since there was no recovery of H^+-ATPase activity 3 h after diclofop-methyl treatment in either species and there was no further inhibition, suggesting that inhibition was immediate. It is not clear whether diclofop-methyl inhibits the H^+-ATPase enzyme directly or indirectly through the generation of lipid peroxides and oxygen free radicals. Previously, a collapse in the transmembrane proton gradient has been attributed to diclofop-methyl acting as a proton ionophore, thus removing control of the transmembrane proton gradient from the enzyme rather than inactivating the enzyme itself.[37] In the field, cyclohexanediones cause similar symptoms to aryloxyphenoxypropionates, suggesting that they may have a common site of action. However, although cycloxydim did inhibit H^+-ATPase activity in sugar beet, it had no effect in black-grass and sethoxydim had no effect in either species.

Auxin-type herbicides did not inhibit or increase the H^+-ATPase activity of isolated plasma membrane vesicles, although there was a slight stimulation of activity by 2,4-D in sugar beet. In other systems auxin-type herbicides are known to promote H^+-pumping out of the plant cell through an activation of the H^+-ATPase and have been linked to an increased rate of elongation growth.[38] An in-vivo treatment of tobacco (*Nicotiana tabacum* L.) with auxin for 24 h promoted a 100-fold increase of the in-vitro auxin sensitivity of the plasma membrane H^+-ATPase.[39] Activation of H^+-ATPase activity is known to antagonise the action of aryloxyphenoxypropionates such as diclofop-methyl, presumably at or near the plasma membrane.[14] The mechanism of this antagonism is unclear, but it is likely that auxin and 2,4-D stimulate the H^+-ATPase to repolarise the membrane, re-establish net transmembrane acidification and restore cytoplasmic pH to homeostasis. Any diclofop-methyl-induced senescence is therefore reversed or delayed.[13]

Clearly, there are differences in the response of the plasma membrane H^+-ATPase to several classes of graminicides and auxin-type herbicides. A high degree of inhibition by the thiocarbamates, triallate and diallate, suggests that the mode of action of these herbicides may involve an effect at the plasma membrane. Similarly, an effect at the plasma membrane may be important in the mode of action of the aryloxyphenoxypropionates, but, on the basis of these observations, it is less likely with both the cyclohexanedione and chloroacetamide graminicides. This study reports the findings of experiments where herbicides were added directly to the plasma membrane isolated from untreated sugar beet and black-grass plants and as such is an in-vitro study. The findings of H^+-ATPase activity from treated plants will be reported elsewhere.

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